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COMMENTARY

Cyclic Dimeric GMP-Mediated Decisions in Surface-Grown *Vibrio parahaemolyticus*: a Different Kind of Motile-to-Sessile Transition

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Cyclic dimeric GMP (c-di-GMP) is a ubiquitous bacterial second messenger that was discovered over a quarter of century ago by Moshe Benziman and his colleagues at The Hebrew University of Jerusalem (15, 16). The expanding universe of c-di-GMP signaling is dominated by the view that this second messenger controls a limited number of bacterial lifestyle decisions, e.g., the transition of single motile cells to a surface-attached lifestyle, formation and dissolution of biofilms, and, in selected pathogens, the transition from acute to chronic infection. c-di-GMP regulates these transitions by affecting the synthesis and activities of various cell surface components, including flagella, pili, adhesins, and exopolysaccharides and extracellular DNA and virulence factors (6, 14, 19, 22). In this issue, Linda McCarter's group at The University of Iowa and her colleagues at the University of Washington describe how c-di-GMP controls a motile-to-sessile transition of a different kind than the transition of swimming cells to sessility (3). These researchers have begun unraveling molecular details of the c-di-GMP-based decision-making process that takes place in surface-grown *Vibrio parahaemolyticus*. This "garden variety" gammaproteobacterium lives in the sea and occasionally acts as an opportunistic seafood-borne human pathogen (11). The emerging picture is intriguing, sophisticated, and instructive; therefore, it deserves a closer look.

While growing on surfaces, *V. parahaemolyticus* has an option of staying put and forming a structured biofilm or spreading over the surface by swarming, a form of social motility (11, 23). A hallmark of the *V. parahaemolyticus* surface biofilms is the sticky capsular polysaccharide synthesized by the proteins encoded in the *cpsA* locus, whereas a hallmark of surface exploration is a set of lateral flagella encoded by the *laf* genes (1). The lateral flagella are different from the single polar flagellum used for swimming in liquid media. Aside from having different types of flagella, swarming *V. parahaemolyticus* cells also have a distinct morphology from that of liquid-grown swimmers (11).

Earlier, the McCarter group described the ScrC protein as an important player in making the "biofilm versus swarming" decision (4). ScrC is a cytoplasmic membrane-bound enzyme that contains GGDEF and EAL domains arranged in tandem. GGDEF domains are usually associated with diguanylyl cyclase (c-di-GMP synthase) activity, while EAL domains may possess c-di-GMP phosphodiesterase (hydrolase) activity. However, these domains often come in enzymatically inactive forms that have different functions (2, 17). Both domains of ScrC are enzymatically active, which allows this enzyme to either synthesize or to degrade c-di-GMP. It is worth noting that bifunctionality among GGDEF-EAL proteins is rare, and only a few examples other than ScrC have been identified (10, 20).

The *scrC* gene is part of the *scrABC* (swarming and cell surface regulators) operon. The ScrA and ScrB proteins together form a switch capable of turning ScrC from a diguanylyl cyclase, which is the default mode of ScrC in the absence of ScrAB, to a phosphodiesterase (4). When in the phosphodiesterase mode, ScrC lowers intracellular c-di-GMP levels, promotes swarming, and inhibits capsular polysaccharide synthesis. Until recently, it was unknown what triggered the ScrAB switch. This topic was recently addressed in a study by the McCarter group (21), which we briefly discuss here. The second study, by Ferreira and colleagues and published in this issue (3), reveals mechanistic details on how elevated levels of c-di-GMP control capsular polysaccharide synthesis. Future studies are expected to uncover the mechanism(s) through which decreased c-di-GMP levels control swarming.

Let's follow the logic of the study by Ferreira et al. The researchers knew from their earlier work that the *scrABC* deletion results in increased c-di-GMP levels, which repress some *laf* genes and activate the *cpsA* locus genes (4). To explore the sphere of influence of the ScrABC network, they performed whole-genome transcriptional profiling of the wild type and the *scrABC* mutant grown on petri plates. Approximately 80 genes were downregulated in the *scrABC* mutant compared to the wild type. Most of these genes, including all and not just some of the *laf* genes, belonged to the previously characterized category of surface-induced genes (5). The *cpsA* locus genes were among approximately 30 genes whose mRNA levels were increased in the *scrABC* mutant. In this upregulated gene cluster, there were also genes that encode other putative cell surface components, some of which, e.g., the *mfp* operon-encoded membrane fusion proteins, were known to be important for biofilm formation. Thus, the transcriptome profiling experiment solidified the notion that the ScrABC network is the key regulator of the motile (swarming)/sessile (biofilm) choice in surface-grown *V. parahaemolyticus*.

Among genes upregulated in the *scrABC* mutant were two genes that encode peculiar transcription factors, VPA1446 and VP2710 (see Figure 11 in reference 3). Both of these belong to the LuxR/GerE family. The VPA1446 gene, which was designated *cpsQ*, is located between *cpsS* (VPA1447) and the *mfp* operon. CpsS is a third LuxR/GerE family transcription factor that has already been known to repress *cpsA* gene expression (1). Beyond

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their gene linkage, it was striking that CpsQ and CpsS have significant sequence similarity to each other and to VpsT from *Vibrio cholerae*. The latter protein works as a c-di-GMP-dependent transcription regulator that represses swimming and activates exopolysaccharide production in *V. cholerae* (8).

Ferreira et al. analyzed the function of CpsQ and discovered that it works as a direct activator of the *cpsA* locus genes and the *mfp* operon, but it does not affect *laf* gene expression. Interestingly, CpsQ's ability to activate the *cpsA*::*lacZ* reporter fusion in *V. parahaemolyticus* was approximately 2-fold higher in the *scrABC* mutant, which has elevated c-di-GMP levels, than in the wild type. The positive effect of c-di-GMP on CpsQ-dependent activation could be recapitulated in *Escherichia coli*, thus suggesting that c-di-GMP directly affects CpsQ function. This expectation was verified biochemically, as *E. coli* overexpressed and purified His_6::CpsQ protein and was found to contain measurable amounts of bound c-di-GMP, at an approximately 0.1:1 c-di-GMP:protein molar ratio. It is reasonable to expect that some of the bound c-di-GMP was lost during protein purification. While the observed amount of bound c-di-GMP was relatively low, it suggests that CpsQ binds c-di-GMP *in vivo*.

To support the observation that CpsQ binds c-di-GMP, Ferreira et al. (3) mutated Arg134 of CpsQ, corresponding to the residue that interacts with c-di-GMP in VpsT (8). They found that the CpsQ R134A mutant no longer activated *cpsA*::*lacZ* expression and that the purified CpsQ R134A mutant protein no longer contained bound c-di-GMP. Given reasonably high (32%) sequence identity between CpsQ and VpsT, conservation of the c-di-GMP-binding pockets (8), and evidence that at least one of these residues is important for ligand binding, one can assume that both proteins bind c-di-GMP in a similar manner. However, it would be nice at some future point to compare the CpsQ and VspT structures to learn more about the mechanisms of c-di-GMP binding and activation of the LuxR/GerE transcription factors.

Somewhat surprisingly, Ferreira et al. could not reconstitute His_6::CpsQ with c-di-GMP *in vitro*, despite the fact that positive controls involving known c-di-GMP-binding proteins worked fine in their hands (3). The reason for unsuccessful protein-ligand reconstitution remains unclear. One possibility is that the c-di-GMP concentration used in the reconstitution assays was too low to allow appreciable formation of c-di-GMP dimers. It is the intercalated dimer of c-di-GMP molecules that is found between the two protein monomers in the crystal structure of the VpsT dimer (8). High-salt buffers used in protein purification could also have impeded CpsQ reconstitution with c-di-GMP by limiting the conformational flexibility of the CpsQ dimers.

After identifying CpsQ as the primary regulator of *cpsA* gene expression, Ferreira and colleagues focused their attention on genetic approaches to decipher the hierarchy among the *cpsA* regulators. They found that CspS inhibited *cpsA* expression, because it repressed expression of *cpsR* (VP0514). CpsR in turn activated expression of *cpsQ*. CpsQ activates its own gene expression as well. CpsR is a member of the AAA+ family of transcription factors and is similar to *V. cholerae* VpsR and *Pseudomonas aeruginosa* FleQ, both of which are c-di-GMP-binding transcription factors that control flagellar and exopolysaccharide gene expression (7, 18). It is noteworthy that CpsS is a closer ortholog of VspT than CpsQ (49% versus 32% amino acid identity with VspT), has a predicted c-di-GMP-binding motif, and probably binds c-di-GMP. What a byzantine complexity of transcription factors that likely bind c-di-GMP! At present, vibrios have become champions in employing c-di-GMP for transcriptional regulation.

Why *V. parahaemolyticus* needs such a complex regulatory system to control capsule polysaccharide synthesis and why so many transcription regulators of this system apparently depend on c-di-GMP are two unanswered questions. Future studies will hopefully reveal the logic underlying this regulatory puzzle. It is also unknown where the second LuxR/GerE transcriptional factor, VP2710, whose expression was increased in the *scrABC* mutant, fits. Note that VP2710 also contains the predicted VspT-type c-di-GMP-binding motif. Is it involved in *laf* gene expression, or does it control a different set of surface-induced properties? We trust that the McCarter group is looking for answers to these questions.

Let's now turn to the second recent study reported by the McCarter group (21), in which the researchers discovered that ScrABC represents a quorum-sensing system—with a novel autoinducer and a novel network architecture (13). ScrA, a predicted pyridoxal-dependent aminotransferase, synthesizes an autoinducer whose structure is yet to be determined. ScrB, a predicted periplasmic binding protein, binds the autoinducer. It is believed that the ScrB-autoinducer complex, expected to be present at high cell density, interacts with the large periplasmic domain of ScrC and switches ScrC to the c-di-GMP phosphodiesterase mode. A decrease in c-di-GMP levels in turn activates *laf* gene expression (via an as-yet-uncharacterized mechanism) and promotes swarming. At low cell density and low autoinducer concentration, the autoinducer-free ScrB may not interact with ScrC or interact differently, which stimulates the diguanylyl cyclase activity of ScrC. c-di-GMP synthesized by ScrC activates *cpsA* locus genes via CpsQ, which is part of the byzantine CpsS-CpsR-CpsQ cascade.

One can envision that sparse *V. parahaemolyticus* microcolonies (low cell density) grown on a surface of a shellfish (or human intestine) would tend to produce the sticky capsular polysaccharide and stay put. However, when cells are overcrowded, increased levels of the surface-specific autoinducer would stimulate cell differentiation into swarmers and initiate their migration away from the colony. If crowd avoidance on an surface that is otherwise favorable for growth is the goal, then expanding the surface colonization area appears to be a more sensible strategy than swimming away from the surface into potentially more dangerous surroundings, e.g., open sea (or bodily fluids).

The studies discussed above reveal important new insights into the c-di-GMP-mediated decision-making processes for surface-grown bacteria. Most bacteria spend a significant fraction of their lives growing on surfaces, many have c-di-GMP signaling systems, and a number of species display social motility on surfaces. Therefore, revelations from *V. parahaemolyticus* will be informative for understanding surface behavior in other bacteria. Some of the uncovered players will likely be similar. Others may be different, e.g., *P. aeruginosa* also relies on c-di-GMP signaling but does not appear to involve a new quorum-sensing system for the motile-to-sessile transition on surfaces (9, 12).

The McCarter lab papers serve as a reminder that important discoveries can be derived from various "garden variety" bacteria and that a better understanding of the underlying biological mechanisms derived from these discoveries ultimately accelerates research progress for "elite" pathogens. And, of course, neither *V. parahaemolyticus* nor other bacteria come in a so-called "garden variety"; this term is a misnomer. We look forward to seeing McCarter and colleagues paint new pieces of the picture, revealing

an intriguing and sophisticated decision-making process in an avid surface explorer, *V. parahaemolyticus*.

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